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Gata3 directly regulates early inner ear expression of Fgf10

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ABSTRACT

The analysis of Fgf10 mouse mutants has demonstrated a critical role for this ligand in neurosensory development of the vertebrate inner ear, and we have been looking to define the direct upstream regulators of Fgf10 in this sensory organ, as part of constructing the programme of early inner ear development. Through the analysis of reporter constructs in transgenic mouse embryos and neonatal mice, in this report we define a minimal 1400 bp enhancer from the 5′ flanking region of Fgf10. This enhancer drives reporter transgene expression in a manner that recapitulates endogenous expression of Fgf10, from its initial onset in the invaginating otic placode and onwards throughout gestation, controlling Fgf10 expression in all developing sensory patches and in the developing VIIIth ganglion. This regulatory region includes three putative Gata3 binding sites that we demonstrate directly interacts with Gata3 protein through the DNA binding domain with differing affinities. Site directed mutagenesis of all three sites and functional testing in transgenic embryos using reporter transgenes reveals an absolute requirement for Gata3 in controlling Fgf10 expression. Transgenic analysis of individual Gata3 binding site mutations illustrates that only one of these binding sites is necessary for reporter expression. Together these data demonstrate that Gata3 directly activates Fgf10 in the early inner ear, and does so through a single binding site.

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Introduction

Fibroblast growth factors (FGFs) play multiple roles during development of the mammalian inner ear. During induction of the inner ear, competent surface ectoderm adjacent to hindbrain rhombomeres 5 and 6 thickens to form the otic placode that subsequently invaginates to form the otocyst (Kiernan et al., 2002; Ohyama et al., 2007; Torres and Giraldez, 1998). Two ligands, FGF3 and FGF10, have been shown to be redundantly required for induction of the mouse otocyst (Alvarez et al., 2003; Wright and Mansour, 2003), but an additional requirement for FGF8 as well (Dominguez-Frutos et al., 2009; Ladher et al., 2005; Zelarayan et al., 2007) indicates that the action of multiple FGFs, derived from different periotic tissues, the hindbrain, mesoderm and endoderm, are all required in concert for the early formation of the inner ear (reviewed in (Schimmang, 2007)). The complexity of multiple FGFs that are expressed near the forming otic placode has necessitated double and compound mutant analyses, together with misexpression studies, to fully discern the distinct role(s) individual FGFs play in inner ear induction. However, less attention has been focused on any later redundant roles that may also exist in the developing inner ear, since a number of FGFs are also expressed within the developing otic epithelium. Nevertheless, additional and unique roles for both mouse Fgf3 and Fgf10 in the otocyst itself have also been reported. Ligands from both genes are expressed in the antero-ventral otocyst, in a prosensory/neurogenic region where both sensory cells and neuroblasts are born (reviewed in (Fekete, 1996; Fekete and Wu, 2002)). This expression is consistent with the finding of a reduced vestibulo-acoustic (VIII) ganglion in Fgf3 mutants (Hatch et al., 2007; Mansour et al., 1993). In Fgf10 mouse mutants, defects in both vestibular sensory neurons and hair cells have been reported (Pauley et al., 2003); the posterior crista is absent, with the horizontal and anterior cristae reduced and disoriented, and whilst vestibular innervation is initiated normally, nerve fibres fail to target the missing crista, and innervation is reduced to the remaining cristae (Pauley et al., 2003). Thus expression of FGFs 3 and 10 in the otocyst is required for proper development of the otic epithelium in a non-redundant manner. Additional FGFs have also been shown to be critical for the normal development within the otic epithelium. Both Fgf8 (Jacques et al., 2007) and

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Fgf20 (Hayashi et al., 2008; Huh et al., 2012) are involved in the specification of distinct cell types in the developing organ of Corti. Thus multiple FGFs exerting discrete roles within the same epithelium suggests a complex upstream regulatory network that ultimately needs to discriminate the transcriptional control of ligand expression in different cells to execute cell-specific functions.

In other vertebrate species, FGF expression domains within and surrounding the developing inner ear suggest that the FGF roles described above are likely to be conserved across species, as has been shown to be the case for the conserved role of FGFs in vertebrate inner ear induction, but the particular FGF members involved in different phenotypes may be different between species. Considering only those roles separate to induction of the inner ear, FGF3 in the zebrafish appears to be responsible more globally for anterior patterning of the otocyst (Hammond and Whitfield, 2011; Hans et al., 2007, 2004; Hans and Westerfield, 2007; Liu et al., 2003). Zebrafish FGFs 3 and 8 are able to impose competence on epithelia to respond to forced expression of Atoh1 to adopt a sensory fate (Sweet et al., 2011). In the chick as in the mouse, FGFs are also involved in the normal formation of semicircular canals. Ectopic FGF3 and FGF10 expression leads to canal dysmorphogenesis in the chick (Chang et al., 2004), whereas Fgf10 loss in the mouse leads to loss of the posterior canal (Pauley et al., 2003). Earlier experiments have shown that FGF2 is also able to influence neuronal development of the inner ear. Chick otocyst explant cultures were used to uncover a role for FGF2 in migration and differentiation of cells in the vestibuloacoustic ganglion (Hossain et al., 1996) and FGF2 is able to promote the survival of cochlear neurons in vitro (Carnicero et al., 2001). In vivo in the developing chicken inner ear, ectopic FGF2 and FGF8 increase the size of the vestibulo-acoustic ganglion through further recruitment of cells to the neuroblast lineage (Adamska et al., 2001). More recently, using chick vestibulo-acoustic ganglion explant cultures, FGFs 8, 10 and 19 were all able to promote directional neurite extension (Fantetti and Fekete, 2012). Together these experiments indicate that the expression domains of different FGFs in the otic epithelium are associated with different developmental roles in the inner ear.

Whilst knowledge of the detailed roles of FGFs in the inner ear has advanced dramatically over recent years, our understanding of the upstream molecular mechanisms linking FGFs to the early developmental programme of the inner ear remains rudimentary. Inner ear Fgf10 expression has been shown to be downregulated in both Lmo4 (Deng et al., 2010) and Gata3 (Lillevali et al., 2006) mouse mutants. However, the relationship of Fgf10 to these genes has not yet been defined. We have been looking to define how inner ear Fgf10 expression is directly regulated, and thereby integrate the control of Fgf10 into the molecular programme of inner ear development. Previous studies on Fgf10 regulation had separated regulatory regions governing limb expression (Sasak et al., 2002) from regions regulating some aspects of inner ear expression (Ohuchi et al., 2005). This latter study indicated that the cis-acting DNA regions controlling early inner ear expression are all localised within 7 kb of the transcriptional start site, thereby delimiting this region through which the upstream regulators directly interact to control otocyst expression. In this study we take a transgenic mouse approach to define a minimal enhancer that controls otocyst expression of Fgf10. Further, we show that the transcription factor Gata3 is a direct upstream regulator of Fgf10 that activates inner ear transcription through one of three Gata3 binding sites. We also show that Fgf10 expression is subject to control through retinoic acid (RA) signalling, and reveal that administration of additional RA leads to down-regulation and/or dorsal displacement of Fgf10 expression.

Materials and methods

Generation of transgenic mice

Transgenic mice were generated by pronuclear microinjection of linearised DNA constructs into pronuclei of F2 eggs produced by superovulation of 28–38 day old F1(CBAxC57Bl/6) females crossed with F1 stud males as described in Hogan et al. (1994). Injected embryos were transferred to pseudopregnant F1(CBAxC57Bl/6) females. Founder transgenic embryos were either sacrificed during gestation or left to go to term to generate stable reporter lines for subsequent analysis. Genotyping for the presence of the reporter transgene was by using internal primers directed against the ZsGreen fluorescent reporter cassette using yolk sacs (transient embryo analysis) or ear notches as the tissue source for DNA extraction. Primers used for genotyping using PCR were: 5′-GGAGATGACCATGAAGTACCGCATGG-3′ and 5′-GCTTGTGCTGGATGAAGTGCCAGTC-3′ using the following conditions: 95° C for 2 min; (95° C for 30 s, 65.5° C for 30 s, 72° C for 60 s) for 30 cycles; 72° C for 2 min. The numbers of transgenic mice/embryos analysed as founders are detailed in Fig. 2.

Administration of retinoic acid

Exogenous all trans retinoic acid was administered using a novel non-invasive technique we have recently developed (Cadot et al., 2012). Retinoic acid was mixed with Dr Oetkers brand writing icing sugar and administered to transgenic pregnant mice, at a dose of 25 mg/kg at either 7.75 dpc or 8.5 dpc gestation stages, with analysis carried out at 9.5 dpc.

In situ hybridisation

In situ hybridisation was carried out according to standard methods. For in situ hybridisation probes, cDNA fragments were amplified using Phusion DNA polymerase (New England Biolabs) in hi-fidelity PCR. For Fgf10, sequence from nucleotides 695–1264 corresponding to transcript ENSMUST00000022246 was amplified. For Gata3, sequence from nucleotides 233–1099 corresponding to transcript ENSMUST00000102976 was subcloned into bluescript. Single-stranded DIG-labelled Fgf10 and Gata3 riboprobes were prepared using RNA polymerases T7 and T3. Mouse embryos analysed were both inbred and hybrid embryos from C57Bl6/J and CBA/J matings. At the appropriate gestational age, pregnant mothers were humanely sacrificed and embryos were dissected, rinsed in PBS and fixed in 4% paraformaldehyde in PBS on ice for between 30 min and 2 h, with exact timing dependent on embryo age/size. For wholemount in situ hybridizations, embryos were subsequently rinsed in PBS and dehydrated through a methanol series and stored at −20° C prior to hybridisation. DIG-labelled probes were used for in situ hybridisation as previously described (Nonchev and Maconochie, 1999). For sections, hybridised embryos were embedded in wax and sectioned at 15 μm.

Analysis of fluorescent activity in transgenic mice.

Embryos were fixed in 4% paraformaldehyde, rinsed in PBS and directly analysed by fluorescent microscopy. Alternatively where sectioned material was required, following fixation, embryos were rinsed in PBS and allowed to equilibrate in 30% sucrose in PBS, transferred to OCT embedding medium and frozen. 14 μm sections were taken on a Leica CM1900 cryostat. For whole mount analysis, inner ears were dissected, fixed in 4% paraformaldehyde, washed in PBS and dehydrated through an increasing ethanol series and cleared in methyl salicylate.

Embryos/sections/ears were examined for fluorescence using a Nikon AZ100 stereomicroscope and images captured using either a Nikon DS-Fi1 or DS-QiMc digital camera. Conventional microscopy was on a Nikon SMZ1500 stereomicroscope using bright and dark field illumination. Analysis at higher magnification was on a Nikon Eclipse TE2000-U and Nikon AZ100.

Electrophoretic mobility shift assays (EMSA)

Recombinant GST-tagged full-length GATA3 protein and partial (103aa–200aa) GATA3 protein (lacking the DNA binding domain) were obtained from Abnova (Abnova, Taiwan). 30 ng of protein was incubated with approximately 50,000 cpm (or approximately 1 ng; specific activity 5×10^4 cpm/ng) of [γ-32P]-dATP double-stranded oligonucleotide probe at room temperature for 1 h, in a 10 μl reaction containing: 20 mM Tris, 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 10 μg bovine serum albumin (BSA) and 5% sucrose. DNA-protein complexes were resolved on 8% polyacrylamide gels (acrylamide/bis-acrylamide 29:1) in 0.5xTBE buffer (45 mM Tris–HCl, 45 mM boric acid, 1 mM EDTA) at 200 V for 35 min. Gels were fixed for 15 min in 10% acetic acid, 20% methanol solution, dried, exposed to a phosphoimager screen and analysed using a Storm phosphoimager and the ImageQuant program (Amersham Biosciences).

Oligonucleotides for the three Gata3 binding sites tested were as follows (only one strand is shown for brevity, but the complementary oligonucleotide strand was also synthesised):

Fgf10 Gata3.1 FOR 5′-GACTAATATCACTGGATAGGC-3′
Fgf10 Gata3.2 FOR 5′-CCTCATTACAGAGATATAATCTAC-3′
Fgf10 Gata3.3 FOR 5′-GTGATTCAAATCTATTGGG-3′

Cloning the upstream Fgf10 genomic region

Primers were designed to amplify a fragment larger than the 3-1 fragment (described in Ohuchi et al. (2005)) that supports limited otic expression. Using the primers Fgf10 3f (5′-CAGAGAG-CATAGCTACTAAGGCATCTG-3′) and Fgf10 3r (5′-GCTGTGA-TGTTCGTCACGTTGTTG-3′) in hi-fidelity PCR with Phusion DNA polymerase (NEB) and the following conditions: $1\times98^\circ$ C for 1 min; $30\times98^\circ$ C for 15 s, 63° C for 30 s, 72° C for 105 s; $1\times72^\circ$ C for 5 min, a 1400 bp fragment was amplified. This fragment was cloned into pbluescript SK and the sequence verified. Finally the Fgf10 test sequence was cloned into a reporter plasmid constructed by combining a β-globin minimal promoter subcloned in front of the promoterless ZsGreen vector obtained from Clontech.

Site-directed mutagenesis

The following primers were used for site-directed mutagenesis of the three Gata3 binding sites, numerically ordered in increasing distance from the transcriptional start site:

Gata3-1: 5′-TACATTCATACATTTATGTTAGTGATATCGGGA GAGATTGGATTCCGT-3′.
Gata3-2: 5′-GAGAATGATTCAGGCCTGATTAAGCTTTACATT CATACATTTATGTTAGTG-3′
Gata3-3: 5′-CTCTTTCTTTCAATAAAGATTTAGAATTCGATA GGCTTAGGCCATAGAA-3′

The above primers were used to mutagenise the individual and combined sites using the Quickchange and Quickchange-Multi site-directed mutagenesis kits (Stratagene) with the Fgf10 enhancer cloned above as template. Mutant enhancer sequences were verified by sequence analysis and subsequently cloned into the β-globin minimal promoter-ZsGreen reporter plasmid for transgenic analysis.

DNA sequence analysis

DNA sequences were obtained from the publically available databases www.ensembl.org and www.ncbi.nlm.nih.gov, with annotation and programmes available at these sites. Analysis of DNA sequence for potential transcription factor binding sites was using Matinspector (Quandt et al., 1995) available at www.genomatix.de.

Results

Delimiting an enhancer controlling otic expression of Fgf10

The expression pattern of Fgf10 in the developing inner ear has been well documented by a number of laboratories previously (Alvarez et al., 2003; Pauley et al., 2003; Urness et al., 2011; Wright and Mansour, 2003) and so here we briefly present the details of the initiation of expression that an inner ear Fgf10 enhancer needs to control. Fgf10 expression in the mouse otic epithelium is first initiated around 8.75 dpc (Fig 1A), where expression is first weakly detected in the anterior region of the otic placode, just as it begins to invaginate (Fig. 1A). As the placode sinks below the surface ectoderm to form the otic cup, Fgf10 expression is more pronounced but still remains restricted to the anterior region (Fig. 1B). As the otic cup begins to approach closure around 9.0–9.25 dpc, Fgf10 expression is still in the anterior otic epithelium but extends ventrally (Fig. 1C, 9.25 dpc). Upon closure to form the otocyst, expression is maintained in the anterior otocyst, but lower levels of Fgf10 expression can be seen throughout the otic epithelium (Fig. 1D, 9.5 dpc). The first major morphogenetic changes of the otocyst are the dorsomedial extrusion of the endolymphatic duct and dorso-ventral elongation of the otocyst at 10.0–10.5 dpc, and expression of Fgf10 remains prominent in the anterior ventral otocyst at these stages e.g. 10.0 dpc (Fig. 1E). Sections through embryos hybridised against an Fgf10 antisense riboprobe show that the stronger antero-ventral expression domain throughout early inner ear development is predominantly medial, located on the neural tube side of the otocyst (Fig. 1 F).

In order to identify the cis-acting DNA sequences that govern otic expression of Fgf10, a 7.0 kb region (Fig. 2A), corresponding to construct F in (Ohuchi et al., 2005) was previously shown to contain the regulatory sequences that control both inner ear (Ohuchi et al., 2005) and limb (Sasak et al., 2002) expression of Fgf10. A smaller ~800 bp region within the 7.0 kb enhancer and located approximately 3.0 kb upstream of the transcriptional start site was also tested (construct 3-1 in (Ohuchi et al., 2005); Fig. 2A) and this also controlled some aspects of inner ear expression, although significantly more limited both spatially and temporally. This ~800 bp region did not activate a lacZ reporter gene in transgenic mice until 11.0 dpc gestational age and, furthermore, the transgene was activated in more restricted inner ear domains than the full 7.0 kb region, illustrating that the minimal Fgf10 enhancer controlling inner ear expression includes sequences beyond this fragment.

To identify the direct upstream regulators of Fgf10, bioinformatics using Matinspector (Quandt et al., 1995) of the 7.0 kb region upstream of exon I was used to analyse this region, and this identified 2194 putative transcription factor binding sites (data not shown). This included binding sites for transcription factors that are known to be active in the early developing inner ear. Of

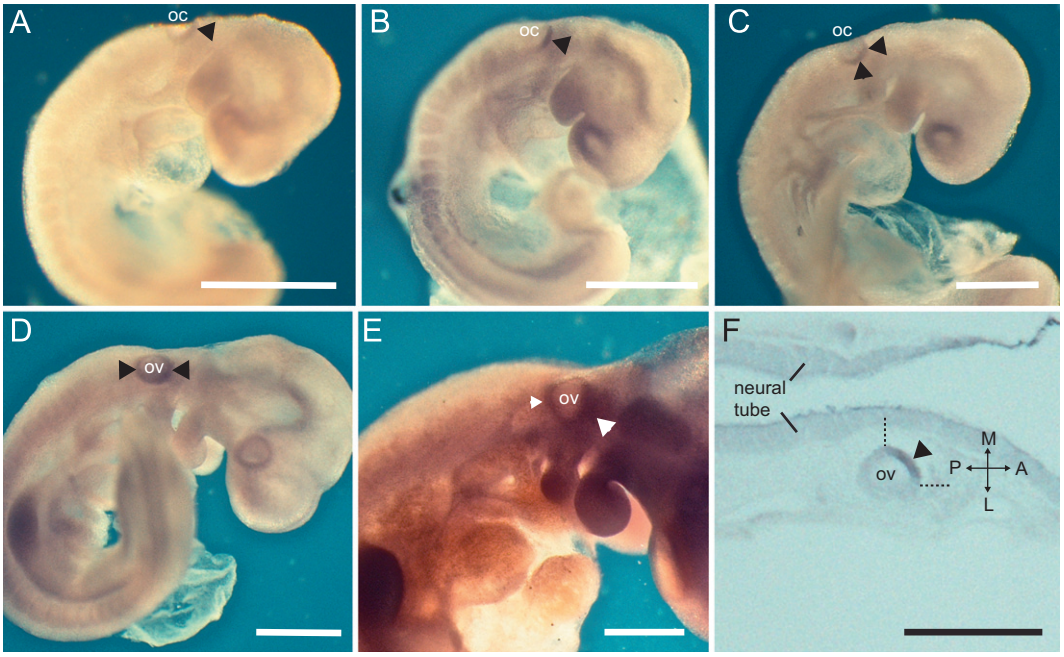

Fig. 1. Initiation of Fgf10 mRNA expression in the developing mouse inner ear. Weak expression in the anterior otic placode at 8.75 dpc (A). Expression is upregulated shortly thereafter as the otic placode sinks to form the otic cup (B), and extends more ventrally in the otic epithelium at 9.0 dpc (C). As the otocyst is formed at 9.5 dpc, expression is throughout the otic epithelium (D), appearing stronger in the antero-ventral region. At 10.0 dpc, these domains are maintained (E). Coronal sections of Fgf10 hybridised embryos at 9.5 dpc illustrate the strong domain of Fgf10 expression is localised to the anterior-medial wall (F). Scale bar 500 μm.

particular interest was the presence of 10 different binding sites for the transcription factor Gata3 in this region (Fig. 2A and Table 1 in Supplementary data). Gata3 is of particular interest as a candidate direct regulator of Fgf10 since Gata3 mouse mutants display significant developmental abnormalities of the inner ear (Duncan et al., 2011; Haugas et al., 2012; Karis et al., 2001), and moreover, Fgf10 expression is downregulated in the Gata3 $-/-$ mutant (Lillevali et al., 2006). However the position of Fgf10 in the hierarchy of targets downstream of Gata3 has not been determined. The presence of multiple binding sites, including clusters of sites around 3.0 kb and 1.0 kb upstream of the transcriptional start, suggested Gata3 as a direct upstream regulator of Fgf10 in the developing inner ear. Given the size of this regulatory region (7.0 kb), binding sites for many other potential transcription factors are also present, and the occurrence of the large number of Gata3 sites in this region prompted us to first delimit the minimal enhancer governing inner ear expression of Fgf10, from its initiation in the inner ear and including all its early otic domains as detailed in Fig. 1.

The 800 bp region controlling later aspects of inner ear expression was used as a basis to design a series of fluorescent reporter constructs containing Fgf10 flanking regions of increasing size. First a 1.4 kb region was tested (construct 266) that extended the ~800 bp region in both 3′ and 5′ directions (Fig. 2 A, B) and was used to generate transgenic embryos. This construct was able to recapitulate the entire endogenous Fgf10 inner ear expression domain ($n=2/3$), including the early domain absent from the 800 bp region. Thus the additional series of reporter constructs with increasing Fgf10 flanking region were not tested, as the 1.4 kb region delimits the minimal functional inner ear enhancer.

The 1.4 kb minimal enhancer controls the complete endogenous Fgf10 inner ear expression pattern.

Two independent transgenic reporter lines for the minimal enhancer were generated and the developmental profiles of reporter expression were compared to one another as well as to endogenous expression of Fgf10 (Fig. 1 and references cited above). The first few cells positive for reporter expression can be detected in the early invaginating otic placode (Fig. 3A), and reporter expression rapidly becomes upregulated and includes further placodal cells as the placode sinks to form the otic cup (Fig. 3B), where expression is seen to occupy the anterior region of the otic epithelium. This corresponds to the endogenous expression of Fgf10 during its initiation (Fig. 1A–C) illustrating this enhancer contains the control elements required for the onset of endogenous expression. As the otic cup approaches closure at 9.25 dpc, expression remains anteriorly localised (Fig. 3C), and a dorsal wholemount view reveals reporter expression is in the medial wall of the otocyst (Fig. 3D), as is the case for endogenous Fgf10 mRNA expression (Fig. 1F). At the early otocyst stage, reporter expression begins to be activated elsewhere in the otic epithelium (Fig. 3E, 9.5 dpc), although expression remains stronger in the antero-ventral otocyst. Reporter expression remains pronounced in the ventral otocyst as the endolymphatic duct begins to bud (Fig. 3F, 10.0 dpc). The endolymphatic duct is clearly discernable at 10.5 dpc, and reporter expression remains prominent in the antero-ventral region, but with a second weaker area of expression in the posterior vesicle (Fig. 3G). This second domain of otic expression corresponds to that of endogenous Fgf10 mRNA (Fig. 1F). Coronal sections through 9.5 dpc embryos at ventral- (Fig. 3H, I) and mid-otocyst levels (Fig. 3J) illustrate reporter expression is at higher levels in the medial wall, with some areas of the lateral wall completely devoid of reporter activity; in general reporter expression appears lowest in posterior-lateral regions of the otic epithelium. The ventral sections (Fig. 3H, I) also illustrate the initial migration of isolated neuroblasts from the anterior ventral wall of the otocyst to populate the vestibulo-acoustic ganglion, positioned just anterior to the otocyst (arrowheads, Fig. 3H, I). This migration of Fgf10 expressing neuroblasts becomes clearer in sections at 10.5 dpc after more extensive migration of FGF10 expressing cells has occurred; in embryo sections through the ventral otocyst (Fig. 3K) Fgf10 positive cells are abundant in the developing vestibulo-acoustic ganglion (VIIIth cranial ganglion), in addition to the

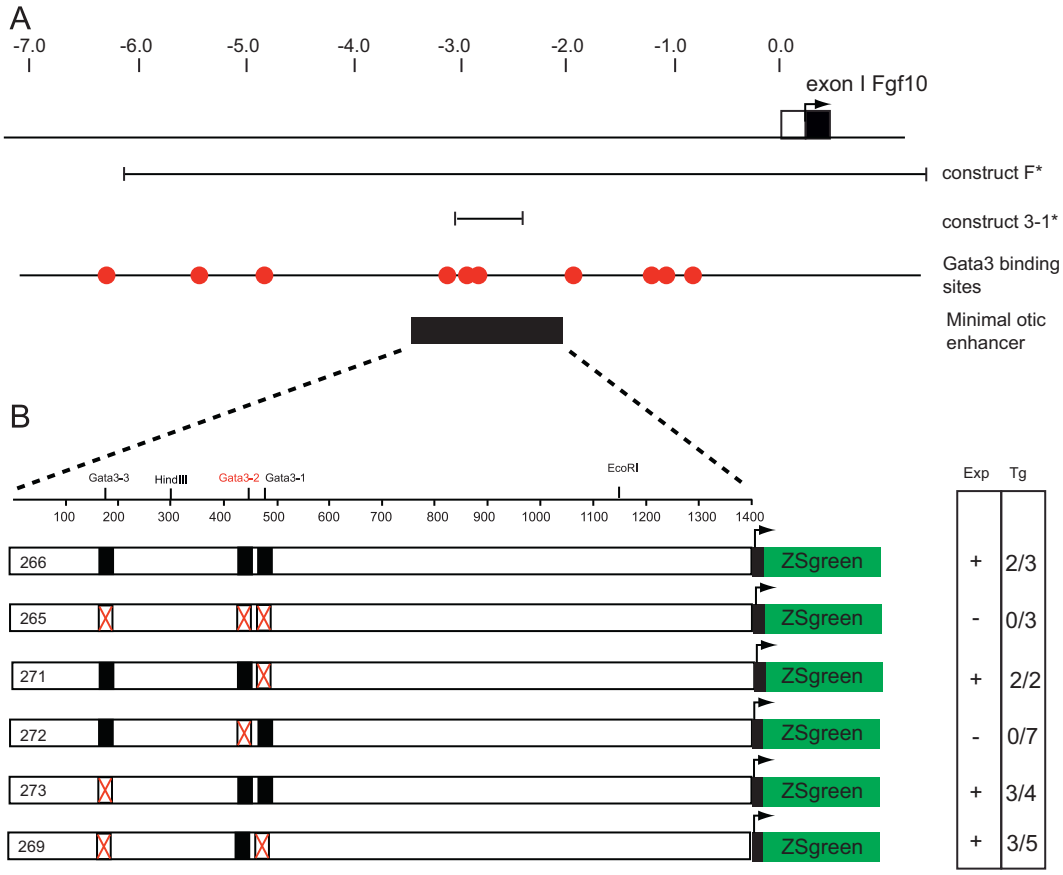

Fig. 2. Schematic of the Fgf10 upstream region and constructs tested. A. Overview of the location of fragments previously tested in (Ohuchi et al., 2005) and highlighted with an asterix, and the location of the ten putative Gata3 binding sites in the 7.0 kb region that controls endogenous Fgf10 inner ear expression. (B) Constructs generated and tested in this report. Black filled box represents wild-type and red-cross box mutated Gata3 binding sites. Gata3 sites numbered consecutively moving away from the promoter. Table to the right summarises presence or absence of inner ear expression +/− (column Exp), with numbers of expressing embryos/transgenic embryos detailed in the column labelled Tg. Pertinent restriction sites indicated above.

anterior and medial walls. In more dorsal sections (Fig. 3L), the VIIIth ganglion is absent as is reporter expression outside the otic epithelium, but expression in the medial wall as well as two more patches of reporter expression are evident at anterior and posterior locations (Fig. 3L), that likely correspond to the developing cristae (see below). In a second independently generated transgenic line, an additional area of early reporter expression was noted. In this second line, transgene integration lead to weaker expression in the developing inner ear, although clearly evident in the ventral otocyst at 9.5 dpc (Fig. 3N), and transverse sections reveal expression in the ventral wall as well as the developing vestibulo-acoustic ganglion (Fig. 3O). However for this line, expression was also detected specifically in developing hindbrain rhombomere 4, being initiated around 8.75 dpc (Fig. 3M) and upregulated by 9.0 dpc, and clearly evident in the ventral neural tube (Fig. 3O). A dorsal view at 9.5 dpc shows strong rhombomere 4 reporter activity, just anterior to otocyst expression (Fig. 3P), with the otocyst developing alongside rhombomeres 5 and 6. This hindbrain expression is an endogenous area of Fgf10 expression (Alvarez et al., 2003) and indicates elements controlling this region of endogenous expression are also present in the minimal otic enhancer described here.

We also followed reporter expression during later stages of inner ear development, as although the smaller 800 bp Fgf10 genomic fragment was shown to initiate reporter expression at E11.0 (Ohuchi et al., 2005), additional aspects of endogenous inner ear expression were not under the control of this regulatory region, and we wanted to investigate whether the minimal enhancer described here also governs activity of these missing domains. At 11.5 dpc, reporter transgene activity was still clearly visible in the inner ear in whole mount embryos as three prominent patches of expression (Fig. 4A), corresponding to the developing cristae, together with more ventral expression that could not be as clearly visualised through the overlying tissue layers. At 13.5 dpc, inner ears were dissected away from embryos, and reporter expression was now very evidently localised to all six sensory patches; in the three cristae, the utricular and saccular maculae as well as the sensory epithelium of the developing cochlea (Fig. 4B). Sections through the 13.5 dpc inner ear at different levels (Fig. 4C) confirmed expression within the sensory patch present in the crista (Fig. 4D), in the maculae (Fig. 4E, F) as well as throughout the sensory epithelium of the cochlea (Fig. 4F, G). Furthermore reporter expression was also evident in cells in the spiral ganglion (Fig. 4F, G) and the vestibular ganglion (Fig. 4E), consistent with the earlier expression seen in delaminating neuroblasts in the otocyst. At 17.5 dpc, terminal differentiation of future sensory hair cells begins. Fgf10 reporter expression was seen in all developing sensory patches, the spiral and vestibular ganglion in whole mount ears (data not shown) and these areas of expression are still evident at P0 (Fig. 4H), with prominent reporter expression in the three cristae, two maculae and the coiled cochlea (Fig. 4H). Sections of P0 heads of transgenic mice showed reporter expression localised to the sensory epithelia of the cristae (Fig. 4I) and the maculae (Fig. 4I, J) and in the organ of Corti, reporter expression was present in the greater epithelial ridge, but absent in the adjacent lesser epithelial ridge

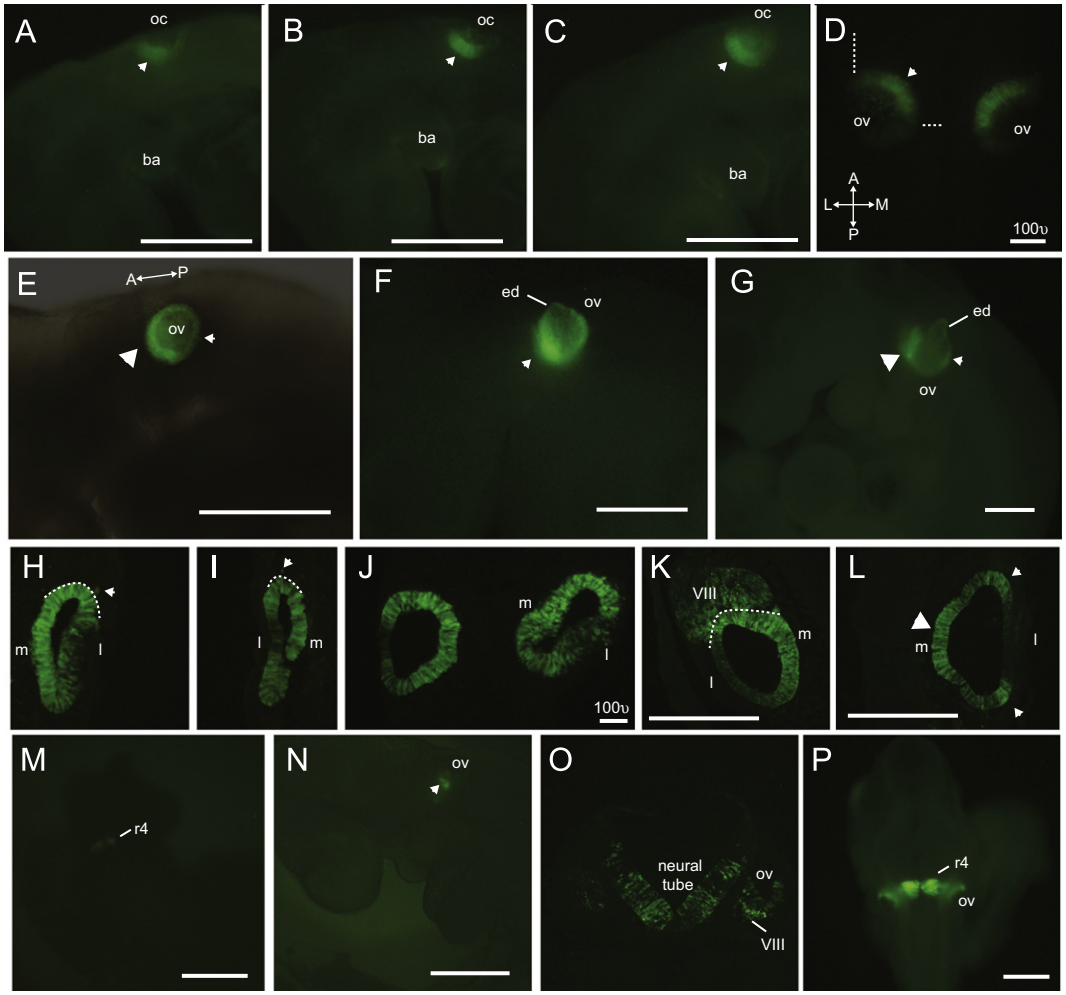

Fig. 3. Early reporter activity of the 1.4 kb Fgf10 enhancer in the developing inner ear. Reporter activity first detected around 8.75 dpc (A) and is rapidly upregulated as the placode sinks to form the early otic cup (B) and is maintained in this region at 9.25 dpc as the otic cup approaches closure (C). (A–C) anterior is to the left. Dorsal wholemount view illustrates reporter activity in the anterior-medial wall (9.25 dpc, D). (E–G; anterior to the left). At the early otocyst stage, expression is also detected elsewhere in the otic epithelium (9.5 dpc, E) albeit weaker than the antero-ventral domain, and is maintained at 10.0 dpc (F). Strong anterior and weaker posterior domains of expression at 10.5 dpc (G). Coronal sections at ventral (H, I) and mid-otocyst (J) planes through the 9.5 dpc otocyst illustrate high levels of activity in the medial and anterior walls. Isolated cells leaving the otic epithelium highlighted by arrowhead. Ventral (K) and mid-otocyst (L) sections through the 10.5 dpc otocyst reveal reporter activity in the medial wall and two patches in the anterior and posterior wall (small arrowheads), as well as post migratory neuroblasts postitive for reporter activity in the VIIIth ganglion (K). A second independent transgenic line shows the same expression domains although weaker, with additional reporter activity detected in the developing hindbrain. Hindbrain expression first detected at 8.75 dpc (M, anterior-top), and is strongly upregulated in rhombomere 4 by 9.5 dpc (P, anterior-top). Reporter activity is detected in the ventral otocyst at 9.5 dpc (N) and transverse sections reveal reporter activity in the VIII ganglion (10.0 dpc, O). ba-branchial arch; oc-otic cup; ov-otocyst, ed-endolymphatic duct, m-medial, l-lateral. Scale bar 500 μm unless otherwise indicated.

and stria vascularis (Fig. 4K). Expression was also present in the spiral ganglion (Fig. 4K) as at earlier stages.

These later patterns of reporter expression recapitulate those of endogenous Fgf10 mRNA in the inner ear (Pauley et al., 2003), and together with the analysis of early reporter expression above (Fig. 3) illustrates the 1.4 kb minimal enhancer contains the regulatory regions that control the onset and maintenance of embryonic Fgf10 expression in the developing mouse inner ear throughout gestation.

Expression analysis supports Gata3 as a candidate direct upstream regulator of Fgf10

The definition of the minimal enhancer controlling Fgf10 inner ear expression to 1.4 kb of flanking Fgf10 sequence was next readdressed with respect to the bioinformatic analysis for putative transcription factor binding sites, since this smaller regulatory region will considerably reduce the number of potential candidates. Interestingly, one of the two clusters of Gata3 binding sites highlighted earlier in the 7.0 kb region is located within this regulatory region (Fig. 2A). Since Gata3 has previously been reported to be expressed in the developing inner ear (Lawoko-Kerali et al., 2002; Nardelli et al., 1999; Pauley et al., 2003), we next investigated the details of onset and early otic expression of Gata3 mRNA using whole mount in situ hybridisation. Gata3 expression is initiated in the otic placode at 8.75–9.0 dpc, with expression throughout the invaginating placode (Fig. 5A), and as the placode sinks to form the otic cup (Fig. 5B), Gata3 mRNA expression is located throughout the otic epithelium (dorsal view, Fig. 5C), but is considerably weaker in the lateral wall. Gata3 expression can be seen throughout the closing otic cup (Fig. 5D) although again weaker in lateral regions, and throughout the early otocyst (9.5 dpc, Fig. 5E). Gata3 expression is also evident in the developing vestibuloacoustic ganglion (10.5 dpc, Fig. 5F) located just anterior to the otocyst. Sections through embryos hybridised against Gata3 riboprobes illustrate extensive expression thoughout the otic epithelium (Fig. 5G). Thus through early development of the inner ear, the expression pattern of Gata3

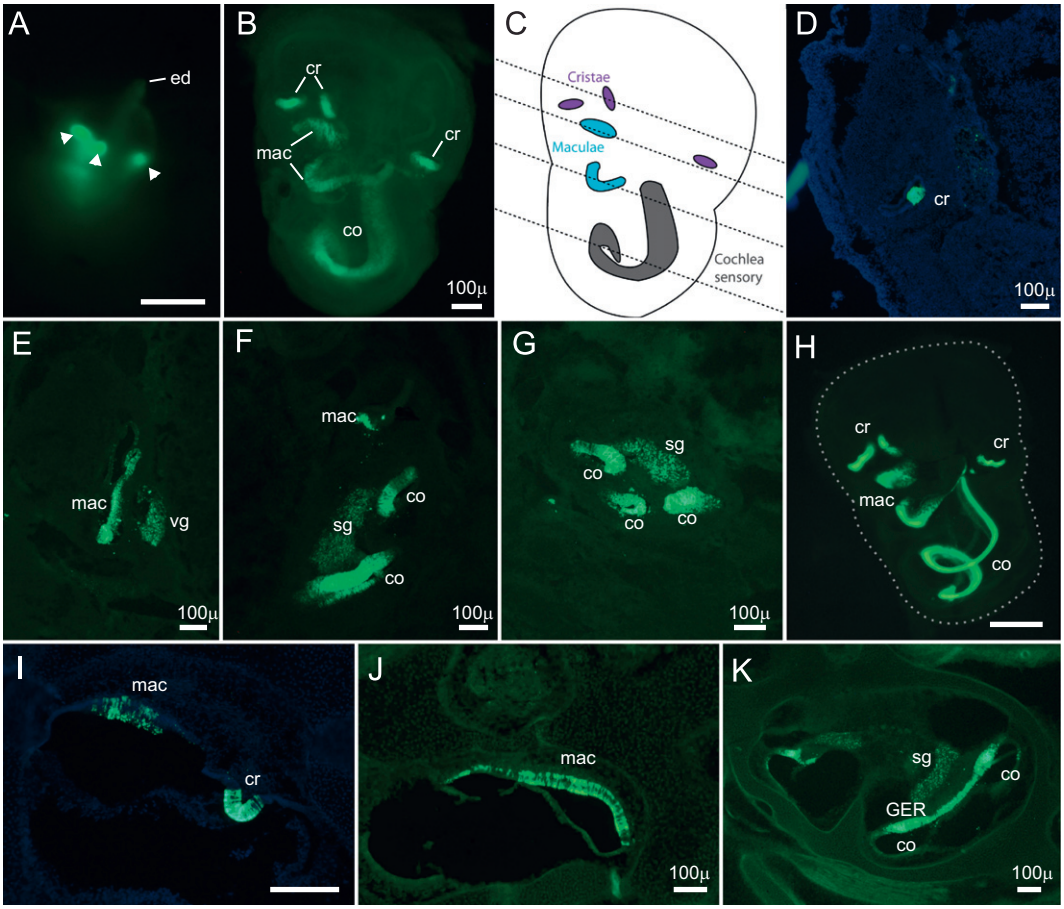

Fig. 4. The 1.4 kb Fgf10 minimal enhancer controls expression in all developing sensory patches through late gestation. Wholemount inner ears at 11.5 dpc reveal reporter expression in two anterior and one posterior equatorial patch corresponding to the separating cristae, as well as in ventral regions (A). Wholemount ears at 13.5 dpc reveal reporter activity in all three cristae (cr), in the utricular and saccular maculae (mac) and in the developing cochlea (co, B). Sections taken as illustrated in (C) of 13.5 dpc heads detail expression in the crista (D), macula (E and F), cochlea (F, G) as well as in vestibular (vg) (E) and spiral (sg) ganglia (F,G). These domains are maintained in P0 wholemount ears (H). Sections taken at P0 illustrate reporter activity remains robust in maculae, cristae and cochlea (I-dorsal, J-mid ear, K-ventral sections). GER—greater epithelial ridge. Scale bar 500 μm unless otherwise indicated.

overlaps the Fgf10 reporter and mRNA expression domains described above.

The Gata3 binding sites in the minimal enhancer are highly conserved

The sequence of the three Gata3 binding sites in the minimal enhancer was further analysed. Regions of sequence homology have been shown to exist between upstream regions of chicken, mouse and human Fgf10 (Ohuchi et al., 2005) (and our unpublished data), and the cluster of these three Gata3 binding sites is within one of these areas of homology. All putative Gata3 binding sites in this cluster were found in the corresponding human, chicken and mouse Fgf10 flanking region. The sequence of Gata3 sites 1 and 2 were found to be completely conserved across all three species (Fig. 6A), whereas Gata3 site 3 showed more interspecific variation, although still represents a potential Gata3 binding site (core similarity 0.88; matrix similarity 0.92; see Table 1, Supplementary data).

Gata3 protein binds all three sites from the Fgf10 minimal enhancer but with differing affinity

These motifs remain notional Gata3 binding sites until tested, so we first investigated the ability of GATA3 protein to bind the three sites, using electrophoretic mobility shift assays (EMSAs). Oligonucleotides were designed that incorporated either sites 1, 2, or 3, and were incubated with full length human GATA3 protein. All oligonucleotides generated a gel-retarded complex in EMSA (Fig. 6B), although the intensity of the complex generated varied between sites, reflecting differing binding affinity of the GATA3 protein for each site. These binding assays were carried out on three separate occasions with identical results. In these assays, no gel shift was noted in the samples lacking GATA3 full length protein (Fig. 6B). The strongest affinity was seen for site 2 (+Gata3 full prot; Fig. 6B), and weaker complexes of similar size were noted for Gata3 sites 1 and 3 (+Gata3 full prot; Fig. 6B).

We next sought to ensure that the interaction with GATA3 protein was through the DNA binding domain of GATA3 and not some other association. Therefore the EMSA reactions were carried out using a truncated GATA3 protein, where the truncation removes this DNA binding domain. In all three cases, the truncated GATA3 protein did not lead to any generation of a gel retarded complex (Fig. 6B; +lanes Gata3 truncated prot). This confirms GATA3 protein binds the Gata3 binding sites via its DNA binding domain.

In order to investigate the specificity of the interaction, we also performed competition EMSAs, where increasing amounts of unlabelled oligonucleotide were incubated along with labelled oligonucleotides and full length GATA3 protein. In the negative control without GATA3 protein, no gel retarded complex was formed (Fig. 6C; −lanes Gata3 prot). Addition of full length protein generated complexes (Fig. 6C,+lanes Gata3 prot) and the

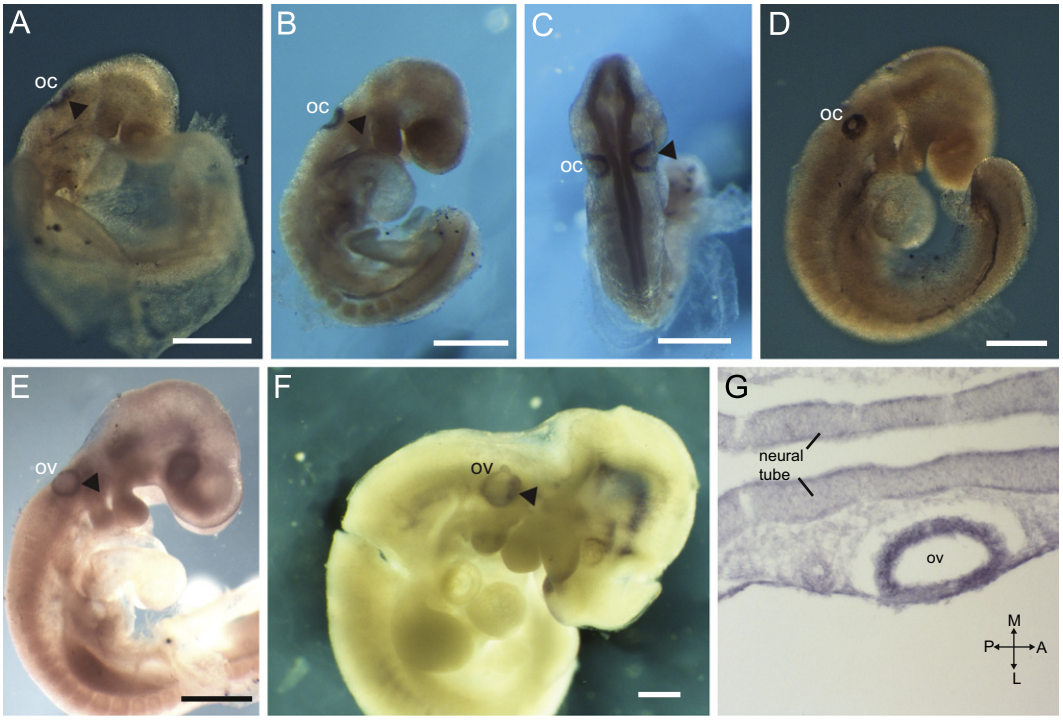

Fig. 5. Early expression of Gata3 mRNA supports its role as a direct upstream regulator of Fgf10. Gata3 otic expression is first seen as the otic placode begins to invaginate at 8.75 dpc (A) and is upregulated as it forms the otic cup (oc) (B). Dorsal views of the otic cup show extensive Gata3 expression in the otic epithelium although absent from the lateral walls (9.0 dpc, C). Expression is maintained as the otic cup approaches closure (9.25 dpc, D) and is also evident throughout the early otocyst (9.5 dpc, E), including expression in the antero-ventral domain (arrowhead). At 10.5 dpc, strong Gata3 expression is maintained in the anterior otic epithelium and developing ganglion (F). Coronal sections at 9.5 dpc illustrate extensive Fgf10 expression in the otic epithelium (G). Scale bar 500 μm.

extra addition of 100x unlabelled oligonucleotides lead to either complete loss (site 1) or dramatic reduction of the gel retarded complex for sites 2 and 3 (Fig. 6C; + × 100) Comp oligo.

Functional analysis of the Gata3 binding sites in vivo

We next sought to address whether the Gata3 sites present in the minimal Fgf10 enhancer have any functional significance. Mutations were generated in all three potential Gata3 binding sites, and the triple mutant enhancer was then cloned in front of the ZsGreen fluorescent reporter gene used earlier. This construct (construct 265, Fig. 2B) was used to generate transgenic embryos. These mutations abolished reporter expression in the otic vesicle and associated VIIIth ganglion (n=0/3, Fig. 7A) in embryos harvested at 10.5 dpc, indicating a requirement for these Gata3 binding sites for reporter activity.

We finally wanted to address the individual or combinatorial requirements of the different Gata3 sites for controlling Fgf10 inner ear expression, and so prepared single and double combinations of mutations for all three sites, and cloned these mutant enhancers into the fluorescent reporter. We first analysed the in vivo function of the single Gata3 mutant sites. Analysis of transgenic embryos at 10.5 dpc harbouring a mutation in Gata3 site 1 (construct 271; Fig. 7B, B′) showed there was no effect on reporter expression (n=2/2). Nor did mutation in Gata3 site 3 lead to loss of reporter expression (construct 273; n=3/4, Fig. 7C, C′). However mutation of Gata3 site 2 alone lead to a complete loss of reporter expression in transgenic embryos at 10.5 dpc, even after repeated injections (construct 272, n=0/7, data not shown). To address any potential combinatorial requirements of sites 1 and 3, reporter transgenes containing combined enhancer mutations in Gata3 sites 1 and 3 were injected, but this too did not lead to loss of reporter expression in the otocyst (construct 269; n=3/5, Fig. 7D–F). Together, these data demonstrate that of the three Gata3 binding sites, only Gata3 site 2 is essential to control Fgf10 expression during early development of the inner ear.

Fgf10 expression is dynamically downregulated by retinoic acid (RA) during the period of hindbrain patterning.

The generation of a stable reporter line for Fgf10 allowed us to investigate the input of other regulatory molecules on Fgf10 expression, such as the developmentally critical signalling molecule retinoic acid (RA). We recently showed that otocyst expression of the related ligand Fgf3 is subject to regulation by RA. Fgf3 is downregulated in a dose dependent manner following administration of exogenous RA, where a dose of 25 mg/kg is able to extinguish Fgf3 expression (Cadot et al., 2012).

To investigate any effects on Fgf10 expression by RA, we used a novel alternative method of feeding RA to pregnant transgenic females carrying the minimal Fgf10 reporter. 25 mg/kg RA was administered at 7.75 dpc, during hindbrain patterning (Fig. 8B–F), and two major changes in the Fgf10 expression pattern were detected when transgenic embryos were analysed at 9.5 dpc. First there was a change in the spatial localisation of reporter expression, with reporter activity shifted dorsally in the otocyst (n=9/21, Fig. 8B, C; compare to control Fig. 8A). Secondly, the antero-ventral expression domain of the Fgf10 reporter was downregulated in the otocyst (n=13/21); expression was either absent in this region (Fig. 8B–D) or not elevated above otic epithelial levels elsewhere (Fig. 8F). Occasionally embryos were detected that retained an anterior patch of reporter expression (n=2/21; Fig. 8E), although this too appeared to be somewhat dorsally shifted. Intriguingly we also noticed reproducible activation of the Fgf10 reporter transgene in anterior regions of the embryo, in the forebrain (n=5/21; Fig. 8B–D).

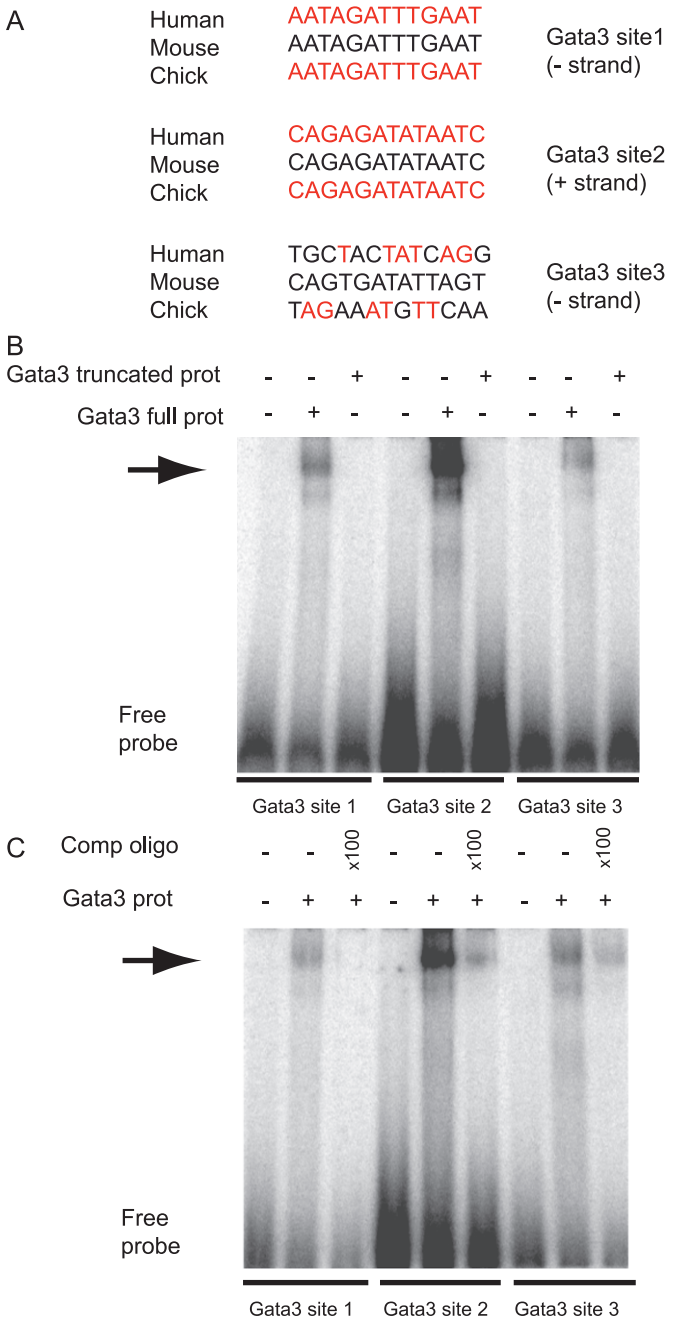

Fig. 6. Fgf10 Gata3 binding sites are highly conserved and bind Gata3 protein in EMSA. (A) Sequence comparison of the mouse 1.4 kb enhancer identifies conserved regions from human, mouse and chick that include Gata3 binding sites. Sequence alignments of the conserved Gata3 binding sites are shown. (B) EMSA analysis of Gata3 binding sites shows full length protein is able to form gel retarded complexes with all three sites but with differing affinities. These complexes are not formed when using a truncated form of the protein that lacks the DNA binding domain. (C) Competition EMSA of the Gata3 binding sites with excess unlabelled oligonucleotides (x100) either reduces or abolishes the gel retarded complex formed with the three Gata3 sites. Gel retarded complexes are indicated by arrows in (B,C).

We next administered 25 mg/kg RA to pregnant transgenic females after the hindbrain was patterned (8.5 dpc), and analysed embryos again at 9.5 dpc. We did not detect any dorsalisation of reporter activity in all the embryos analysed ($n=13$). However in a number of embryos ($n=4/13$), there was some downregulation of the antero-ventral domain of reporter activity (Fig. 8H, I), whereas in most embryos this domain appeared largely unaltered ($n=9/13$; Fig. 8G). In addition, the general epithelial activity in the otocyst also appeared unaltered ($n=13/13$). This illustrates that exogenous RA has the effect to downregulate and change the spatial pattern of Fgf10 expression when applied during the period the hindbrain is being patterned, and also differentially affects expression in the anterior otocyst when administered after hindbrain patterning.

Discussion

Gata3 is a direct upstream regulator of Fgf10 in the developing inner ear.

In this report we delimit the enhancer that controls inner ear expression of Fgf10, shown by the ability of the enhancer to confer activity on a reporter transgene. Detailed analysis of reporter activity reveals that the transgene faithfully recapitulates endogenous Fgf10 expression, from the onset of expression in the inner ear through to neonatal stages. We show the presence of a cluster of Gata3 binding sites in this enhancer, and demonstrate that GATA3 protein binds these sites in vitro through its DNA binding domain. Functional testing of these sites however shows that only one site, Gata3 site 2, is absolutely required for reporter expression. These data indicate that Fgf10 is a direct target for Gata3, and the direct interaction of Gata3 through site 2 is required to activate and maintain expression in the otocyst.

Molecular mechanisms of Fgf10 regulation in the inner ear

Bioinformatic analysis of the 7.0 kb upstream region of Fgf10 revealed the presence of ten Gata3 binding sites, suggesting some sub-organisation of sites in two clusters of binding sites, but the functional analysis here shows only a single Gata3 site is necessary for Fgf10 expression. However this single Gata3 site is not sufficient for otocyst expression since the ~800 bp region previously tested (Ohuchi et al., 2005) was unable to drive reporter expression in the early otocyst. This indicates other inputs in addition to Gata3 and its cognate binding site are required for the initiation of Fgf10 expression in the inner ear. By reducing the DNA region analysed to functionally verified sequence in a minimal 1.4 kb enhancer, we were able to reduce the candidate Gata3 binding sites to just three, and moreover sequence analysis revealed two of the three sites were completely conserved between species. The prediction was that this conservation likely reflected a combinatorial requirement for sites 1 and 2. However the EMSA assays showed that although all three sites bound GATA3 protein, site 2 showed particularly high affinity, in contrast to low affinity interactions with sites 1 and 3, even though one of these sites being completely conserved across species as detailed above. The functional testing of different mutant sites corroborated these in vitro binding data, with only the strong site 2 being absolutely necessary for reporter activity. However it is important to remember this analysis does not rule out a function for the other Gata3 binding sites in Fgf10 regulation, merely that they are not necessary for reporter activity. Nevertheless the relatively poor affinity for binding the GATA3 protein does suggest they are probably not likely involved in executing the Gata3 control of Fgf10 expression. This is somewhat curious given the highly conserved sequence context of site 1. Perhaps this site binds another transcription factor, for example GATA2. Gata2 expression is not initiated in the otocyst until 9.5 dpc (Lillevali et al., 2004), and analysis of Gata2 null alleles did not reveal any inner ear phenotypes up to the time of embryo lethality at 10.5 dpc (Tsai et al., 1994). Perhaps then Gata2 could provide additional support for driving Fgf10 expression later during development through this conserved site 1, although site

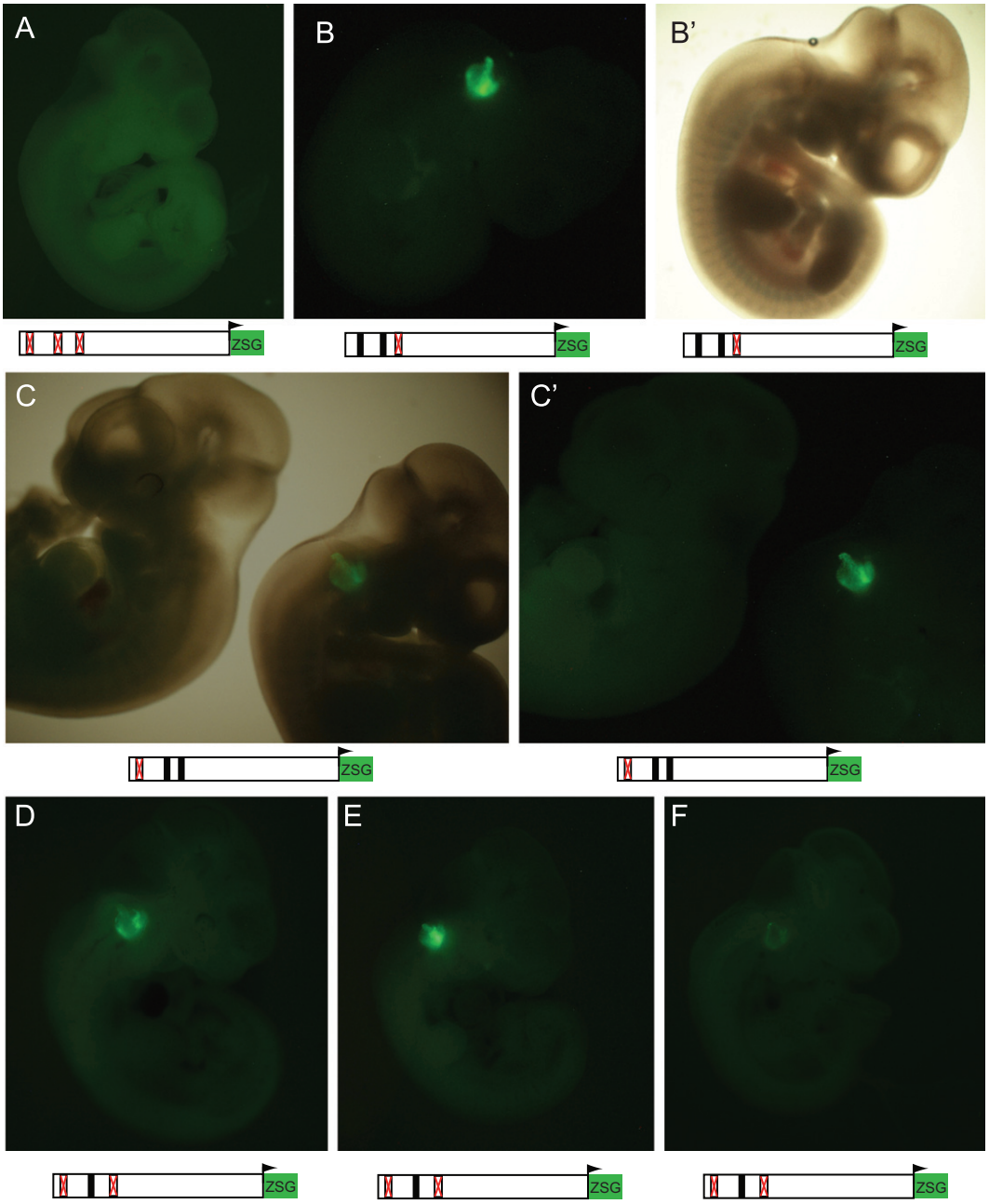

Fig. 7. Gata3 site 2 is essential for in vivo expression of Fgf10. Mutation of all three Gata3 sites lead to loss of activity of the Fgf10 enhancer (A). Mutation in Gata3 site 1 (B) and site 3 (C) did not lead to loss of expression, whereas mutation in site 2 lead to complete loss of reporter activity (not shown). Combined mutations in sites 1 and 3 similarly do not lead to loss of activity (D–F). All embryos analysed at 10.5 dpc, and oriented anterior to the right except C and C′. B′ and C are bright field images corresponding to panels B,C′. In C two transgenic embryos are shown, one showing expression and one without.

1 is not required for early expressions shown in this report. This might suggest a complex interplay of Gata2 and Gata3 controlling Fgf10 inner ear expression during later stages of inner ear development, and indeed Gata2 has been shown to have non-redundant functions during later stages of development in the inner ear. Conditional inactivation of Gata2 was used to overcome the lethality of null mutants and revealed a role for this transcription factor in canal morphogenesis and removal of periotic mesenchyme (Haugas et al., 2010). Arguing against a role for Gata2 in the regulation of Fgf10 is the absence of elements of the Fgf10 phenotype in Gata2 mutants, although it may well require a different strategy to reveal this, for example by preparing conditional double mutant alleles (Gata3−/−; Gata2−/− deleted in the inner ear) to uncover redundant functions of Gata2/3 achieved through directly regulating Fgf10. It would also be interesting to examine Fgf10 expression in Gata2 mutants, although if there is no change, this may be because of redundant functions that the conditional double mutant will address.

The identification of clustered binding sites may well increase the probability of identifying functionally relevant sites via bioinformatics (Gotea et al., 2010; Lifanov et al., 2003), but in this case only one of the three binding sites in the "cluster" was shown to be necessary for expression. The EMSA turned out to have strong predictive value for the functionality of site 2. An alternative explanation for the failure of generating robust gel retarded complexes with sites 1 and 3 could be that additional cofactors are required along with GATA3 for strong binding to sites 1 and 3, but nevertheless the functional testing in this report

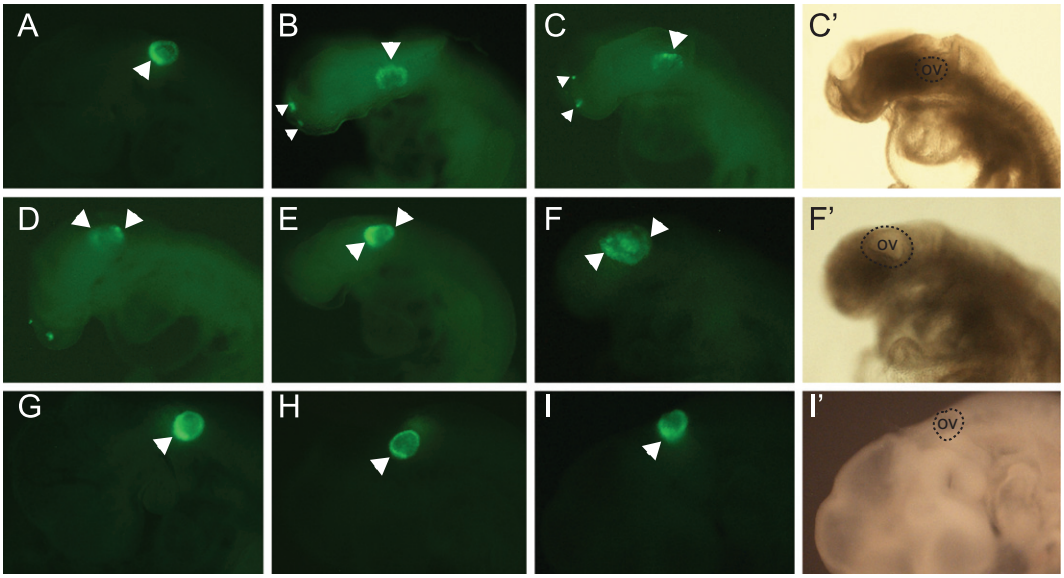

Fig. 8. Response of Fgf10 to retinoic acid. Using the Fgf10 reporter line, the response to 25 mg/kg retinoic acid administered during hindbrain patterning (7.75 dpc; B–F) and after hindbrain patterning (8.5 dpc, G–I) was analysed in embryos at 9.5 dpc. Embryos oriented anterior to left. (A) Control embryos fed pellet without retinoic acid. (B, C) embryos showing dorsalisation of reporter activity and loss of anterior expression; (D, F) embryos exhibiting loss of anterior activity and (E) rarer embryos showing little discernable change in reporter expression. Following RA administration at 8.5 dpc, transgene reporter activity at 9.5 dpc is either unchanged (G) or the antero-ventral domain downregulated to varying extents (H, I). C′, F′and I′ bright field images corresponding to panels C, F and I. ov-otocyst.

shows these sites are not necessary to drive Fgf10 expression. Thus it seems clear that although binding can be achieved with GATA3 protein in vitro for sites 1 and 3, albeit it with low affinity, a functional analysis is absolutely required to determine whether they are required in vivo. To conclude, even a combination of high scores using bioinformatics, a complete conservation of the sequence of sites across three vertebrate species and the ability to bind GATA3 protein in vitro do not necessarily indicate the functional relevance of a Gata3 binding site.

The nature of the additional factors required along with Gata3 to initiate Fgf10 expression in the inner ear remains unknown. Matinspector analysis of the 1.4 kb enhancer defines 499 individual binding sites, including sites for transcription factors that have been shown to play important roles in early inner ear development such as Six1, Pax2 and others. The discussion above indicates that the presence of binding sites alone is only a first step in the identification of any functional link, since consensus sequences for binding sites are likely to occur by chance randomly throughout the genome that may have no functional significance. However the presence of these sites in a functionally verified enhancer at least puts these sites into a functional context. It will require similar biochemical and in vivo approaches to define what cofactors are required to co-operate with Gata3 to initiate Fgf10 expression. Indeed it may well be that binding sites for the cofactors have not yet been defined, and thus are not present in the transcription factor databases, in which case an extensive deletion analysis of the minimal enhancer may also be necessary.

In one of the independent lines analysed, reporter expression in the hindbrain was noted. Fgf10 expression is also present in the early hindbrain (Alvarez et al., 2003), and moreover Gata3 is expressed in rhombomere 4 (Nardelli et al., 1999; Pata et al., 1999). This suggests that there could be a related mechanism utilising Gata3 in both these embryonic contexts. However the position dependent effects of this hindbrain activity of the transgene suggests there are clear differences in the molecular mechanisms underlying regulation of inner ear and hindbrain Fgf10 expression, and likely reflect the interaction of Gata3 with different cofactors in the two contexts, where the binding sites for hindbrain cofactors may be absent or under represented in the minimal inner ear enhancer defined here. There does of course also remain the possibility of "fortuitous" integration of the reporter transgene into another gene expressed in the developing hindbrain, with the reporter scoring activity of this unrelated gene. However we favour the former hypothesis given endogenous Fgf10 expression here and more than one integration event leading to hindbrain expression. We did not pursue the hindbrain regulation further as the focus of this study was on inner ear regulation of Fgf10, but does suggest that a Gata3 input into Fgf10 regulation may well be conserved in the hindbrain as well.

Finally it is important to remember that the hindbrain and inner ear are only two domains of the overall pattern of endogenous Fgf10 expression. To control the entire Fgf10 expression pattern additional enhancers are necessary. A limb enhancer has previously been reported (Sasak et al., 2002), and Fgf10 expression between 8.25–10.5 dpc in pharyngeal mesoderm that gives rise to part of the developing heart tube (Kelly et al., 2001), a domain not identified in the Fgf10-enhancer reporter expression analysis here, suggests this activity is controlled by a different enhancer as well. Indeed this heart enhancer has recently been localised to intron1 and is under the control of Isl1 (Golzio et al., 2012). Another major area of Fgf10 expression and function is the developing lung, and the presence of lung-related transcription factor binding sites 3 kb downstream of the Fgf10-ATG may underlie the presence of a lung enhancer (El Agha et al., 2012), although the ability of this region to control lung expression has yet to be demonstrated. Thus a picture of multiple enhancers controlling different domains of Fgf10 expression is emerging, that together are responsible for controlling the complete Fgf10 expression pattern.

Differences and similarities in retinoic acid control of Fgf10 and Fgf3 in the inner ear.

We recently demonstrated that following administration of exogenous RA during hindbrain patterning (7.75 dpc), Fgf3 expression is downregulated in the otocyst in a dose-dependent manner (Cadot et al., 2012). Prior to complete downregulation, there is a spatial change in the pattern of Fgf3 expression, with a

progressively posterior shift of the antero-ventral domain with increasing RA dose. The response of Fgf10 shows similarities and differences to Fgf3. When RA is administered at 7.75 dpc, Fgf10 expression becomes more dorsally localised in the otocyst, and the antero-ventral domain appears to be lost or downregulated in most embryos. Thus whilst both Fgf3 and Fgf10 expression are downregulated and exhibit changes in otocyst localisation, the precise spatial changes are different.

Administration of RA at 8.5 dpc after hindbrain patterning, i.e. at a time when retinoic acid no longer changes the identity of the posterior hindbrain (Glover et al., 2006; Wood et al., 1994), did not lead to a posterior shift of Fgf3, but rather, only to increasing downregulation of the antero-ventral domain with increasing levels of RA (Cadot et al., 2012). In a similar manner, there was no spatial shift in the otocyst domain of Fgf10 expression. However, there were differences in the extent and incidence of the downregulation observed. Whereas 25 mg/kg RA lead to $>90\%$ embryos showing downregulation of Fgf3 expression (Cadot et al., 2012), only $\sim 30\%$ Fgf10 reporter embryos demonstrated some downregulation. For both genes, the antero-ventral domain is downregulated, with the Fgf10 otic epithelial activity outside this domain remaining unaffected.

Thus plasticity of otocyst patterning is very evident at 7.75 dpc, since both Fgf3 and Fgf10 domains can be relocated in the otocyst although to different locations, whereas administration at 8.5 dpc merely downregulates the antero-ventral domains. Not only does this represent a clear difference in the regulatory response of the two ligands to RA, but also demonstrates the 7.75 dpc inner ear is able to reprogramme its spatial gene expression profile. Whilst we do not know when the different otic axes are determined in the mouse, otocyst transplantation experiments in the chick have shown that Pax2 expression can be reprogrammed to its new axis following rotations performed as late as the otocyst stage (Hutson et al., 1999). Furthermore, retinoic acid appears to be responsible in part for the AP polarity of the otocyst (Bok et al., 2011), with endogenous retinoic acid sources present at both anterior and posterior poles of the otocyst (Cadot et al., 2012). One major retinoic acid source in the otic region is the presomitic/early somitic mesoderm (Romand et al., 2006a) located posteriorly to the developing inner ear, and a second source is the anterior/dorsal otic epithelium itself (Romand et al., 2006b). Administration of excess retinoic acid leads to increases in the amounts of RA at both anterior and posterior poles and affects the spatial organisation of RA responsive genes such as the FGFs during hindbrain patterning.

Fgf10 downregulation with high doses of RA, confirms our earlier studies using RT-PCR to analyse otocyst mRNA (Frenz et al., 2010). What the in vivo study here clearly shows is that it is the antero-ventral domain of Fgf10 expression that is downregulated or lost with exogenous RA administration, in that population of otic cells that will give rise to the vestibuloacoustic ganglion as well as sensory hair cells.

The subtly different spatial responses of Fgf3 and Fgf10 to RA suggests differences in the regulation of the two ligands. This might also reflect differences in their downstream functions within the otic epithelium that have yet to be fully defined with respect to one another, since the potential of Fgf3/10 redundancy within the otic epithelium and the associated vetibuloacoustic ganglion has not yet been reported. The major spatial reprogramming of Fgf3/10 expression to exogenous RA occurs during hindbrain patterning. Whether this response is direct through the FGF otic enhancers or secondary and due to alterations in hindbrain patterning will be interesting to define. In addition this study raises the possibility that some of the changes in otic Fgf10 expression with RA may be mediated through a mechanism that may involve the relocalisation of Gata3, although downregulation of Gata3 as the central mechanism is unlikely as Gata3 mRNA does not appear to be reduced following RA administration (Frenz et al., 2010).

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.11.028.

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